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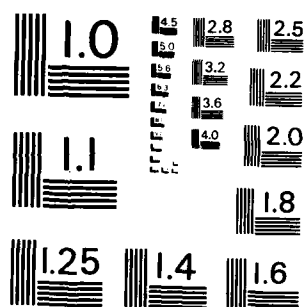
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CELL AND TISSUE ATTACHMENT AND INVASION
OF N. GONORRHOEAE PHENOTYPIC COLONY VARIANTS

Final Scientific Report

GEO. F. BROOKS, M.D.
JOHN F. JAMES, PH.D.

April, 1980

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20314

Contract No. DAMD-17-79-C-9021

University of California San Francisco
San Francisco, California 94143

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20. ABSTRACT

A feasibility study to determine if Langmuir isotherm analysis could be used to study the attachment of Neisseria gonorrhoeae to human tissue culture cells was completed. Requirements for isotherm analysis (equilibrium, reversibility, sub-saturation conditions) were met. The technique demonstrated that gonococcal colony phenotypes differ in their attachment potential for different eukaryotic cell lines. Factors other than pili appeared to function as attachment mediators; these possible attachment mediators remain of unknown composition. Attachment appeared to be very pH dependent attaching best at slightly alkaline conditions. The role of semen and cervical secretions in the attachment process was investigated. Seminal plasma significantly reduced gonococcal attachment to tissue culture cells. Cervical secretions obtained from control women (no history of gonococcal infection) and test women (recent positive cervical culture) behaved differently in effect on gonococcal attachment. Test secretions significantly promoted the attachment of heavily piliated transparent phenotypes, while control secretions significantly blocked the attachment of heavily piliated opaque phenotypes. Scanning electron microscopy of human uterine cervical and fallopian tube tissue explants demonstrated significantly greater attachment of transparent phenotypes to epithelial surfaces. Attachment to cervical tissue was minimal on the squamous cells of the ectocervix and maximal on the transitional and columnar cells of the endocervix. Attachment to fallopian tube epithelia was limited to the microvillus processes of non-ciliated epithelial cells.

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FINAL REPORT

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SUMMARY

A feasibility study to determine if Langmuir isotherm analysis could be used to study the attachment of Neisseria gonorrhoeae to human tissue culture cells was completed. Requirements for isotherm analysis (equilibrium, reversibility, sub-saturation conditions) were met. The technique demonstrated that gonococcal colony phenotypes differ in their attachment potential for different eukaryotic cell lines. Factors other than pili appeared to function as attachment mediators; these possible attachment mediators remain of unknown composition. Attachment appeared to be very pH dependent with opaque phenotypes attaching best at pH 5.5 and transparent phenotypes attaching best at slightly alkaline conditions. The role of semen and cervical secretions in the attachment process was investigated. Seminal plasma significantly reduced gonococcal attachment to tissue culture cells. Cervical secretions obtained from control women (no history of gonococcal infection) and test women (recent positive cervical culture) behaved differently in effect on gonococcal attachment. Test secretions significantly promoted the attachment of heavily piliated transparent phenotypes, while control secretions significantly blocked the attachment of heavily piliated opaque phenotypes. Scanning electron microscopy of human uterine cervical and fallopian tube tissue explants demonstrated significantly greater attachment of transparent phenotypes to epithelial surfaces. Attachment to cervical tissue was minimal on the squamous cells of the ectocervix and maximal on the transitional and columnar cells of the endocervix. Attachment to fallopian tube epithelia was limited to the microvillus processes of non-ciliated epithelial cells.

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FINAL REPORT
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or N. gonorrhoeae Phenotypic Colony Variants
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I. FUNDING AND PERFORMANCE:

The Contract DAMD17-79-C-9021, "Cell and Tissue Attachment and Invasion of N. gonorrhoeae Phenotypic Colony Variants" was funded for the period February 1, 1979, through December 31, 1979. The project was funded as a "feasibility study" without plans for further funding outside of a new proposal.

John F. James, Ph.D. was assigned as a 25% time research collaborator. Claudia J. Lammel Fenner was assigned as a 50% time technician for the project.

Funds from National Institutes of Allergy and Infectious Diseases Grant RO1 AI 15642 were utilized to support portions of this project.

II. WORK DONE:

A. METHODS

1. Neisseria gonorrhoeae Colony Types: N. gonorrhoeae colony phenotypes T1, T2, T3, T4, and opaque and transparent variants were selectively subcultured using a dissecting microscope. The small (T1 and T2) and large (T3 and T4) phenotypes were as described by Kellogg, et al (1,2), and the Op and Tr phenotypes were according to the descriptions of Swanson, and James and Swanson (3,4,5). The following terminology is used in this report:

<u>Kellogg Classification</u>	<u>Terminology used here</u>	
	<u>Piliation*</u>	<u>Opaque/ Transparent</u>
T1	P+	Op/Tr
T2	P++	Op/Tr
T3	P-	Op
T4	P-	Tr

* P+ = piliated; P++ = highly piliated; P- = no pili.

Assessment of piliation was based on previous experience with colony typing and correlating colony types with the degree of piliation demonstrated using negatively stained preparations and electron microscopy techniques. The P+ → P++ represent a continuum rather than definitive colony types with different degrees of piliation. The Op/Tr colony characteristics also appear to be a continuum. We have utilized the more extreme Op and Tr variants for each strain studied. Subcultures of recently isolated strains or cultures from frozen stocks were done daily and isogenic colony variants were maintained. When possible, six colony phenotypes were utilized: P+Op, P+Tr, P++Op, P++Tr, P-Op, and P-Tr.

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) analyses of whole cell *N. gonorrhoeae* lysates were done by the method of Swanson (4). In our laboratory, the 24,000 to 30,000 dalton proteins associated with Op colony phenotypes were comparable to those noted by Swanson.

Neisseria gonorrhoeae strains F62, GC9, 39-7R, MS-11 were laboratory adapted isolates routinely used in our laboratory. Other strains were clinical isolates obtained from male urethral exudate specimens of patients attending local VD clinics. The bacteria were maintained by daily transfer on clear typing agar plates. Media of four peptone compositions were used: 1) modified GC agar base (5); 2) GC agar base (BBL); 3) proteose peptone No. 3 (Difco) GC agar base (15 g proteose peptone, 7.5 g thiotone. All media except GC agar base (BBL) were prepared with the following ingredients: NaCl 5.0 g/l; K₂HPO₄, 4.0 g/l; KH₂PO₄, 1.0 g/l; soluble starch (BBL), 1.0 g/l; Noble agar, Difco, 12.0 g/l. All media contained a laboratory prepared supplement identical in formula to IsovitaleX (BBL). Gonococci were stored at -70° C in Greaves medium or trypticase soy broth (BBL) + 20% v/v glycerol.

2. Radioisotope Labeling of Bacteria: Radioisotope labeled gonococci were prepared by growing the bacteria overnight on colony typing agar plates that had been treated with ³H adenine (adenine {2-³H} 15-30 Ci/mmol New England Nuclear). Each 25 ml agar plate was inoculated with 0.05 ml of ³H-adenine at a concentration 0.5 mCi/ml. The 0.05 ml was streaked over the surface with a glass pipet and allowed to absorb. The gonococci were then transferred from stock cultures to the labeling plates and spread with platinum loops to achieve an evenly spaced colony growth. The ³H-adenine labeled cultures were used after 16-20 hours incubation.

3. Tissue Cell Cultures and Radioisotope Labeling: Flow 2000 cells (Flow Laboratories), a human embryonic lung fibroblast, were obtained from Dr. Ernest Jawetz and Ms. Lavele Hanna, Department of Microbiology, UCSF, or from Flow Laboratories. The cells were cultured in Eagles MEM with Earles balanced salt solution plus 10% fetal calf serum, glutamine and antibiotics. Cells to be used in experiments were dispersed into Leighton tubes with coverslips in tissue culture medium without antibiotics and incubated for 48-72 hours to obtain a monolayer of cells. Initial experiments done with HEp 2, WI-38 and Vero cell lines used similar techniques. Flow 2000 cells were selected for routine use because of availability as well as efficiency as an attachment substrate.

Coverslips with tissue culture cells most often have different numbers of attached cells. To obtain data for standardization of the number of Flow 2000 tissue culture cells on a coverslip, the cells were labeled with L-amino acid mixture ¹⁴C(U) 0.1 mCi/ml (New England Nuclear). The labeling was done in 1 ml of Dulbecco's PBS, pH 7.2, with 10 µL of the ¹⁴C amino acid mixture (final concentration 0.5 µCi/ml). After 1 hour incubation at 37°C, the ¹⁴C amino acid containing mixture was removed and the cells were washed four times with medium 199 without fetal calf serum and used in the attachment assays.

4. Bacterial Adherence: *N. gonorrhoeae* with or without radiolabels were removed from the surface of colony typing agar plates with a dacron swab and suspended to an optical density of 50 Klett (Klett-Summerson Colorimeter, blue filter) in medium 199. The coverslips were removed from the Leighton tubes and placed in 60 mm x 15 mm tissue culture dishes; 2.0 ml of the 50 Klett inoculum or a dilution of this inoculum was added to each Leighton tube coverslip. The

inocula contained about 2.0×10^8 cfu/ml, based on plate counts of colony forming units; the inoculum of a 1:5 dilution of 50 Klett contained about 8.0×10^7 cfu in 2.0 ml for use in visual count analysis of attachment. For Langmuir isotherm analyses (6), additional serial dilutions were made from the 50 Klett suspension and 2.0 ml inocula were used for each of these dilutions.

Plate counts (cfu/ml) of isogenic gonococcal phenotypes vary with the degree of aggregation, so that the same optical density reading (OD or Klett) for different colony phenotypes gives different plate counts. Thus ^3H counts also were made on each of the inoculum dilutions to provide data for standardization of inocula.

The *N. gonorrhoeae*-tissue culture cell mixtures were incubated for 30 minutes at 37°C on a rotating platform shaker. After incubation, the inocula were removed and the coverslips washed once with 2.0 ml of medium 199 to remove non-adherent bacteria. The coverslips were drained of excess wash fluid with a piece of filter paper held to the edge, and then placed in scintillation vials containing 15 ml of Aquasol scintillation fluid (New England Nuclear). Radioactivity (^3H and ^{14}C) was determined by liquid scintillation counting in a Beckman liquid scintillation spectrometer.

For microscopic examination of coverslips, the standard inoculum (1:5 of 50 Klett) was used. The coverslips were incubated and washed as described above. The monolayers were washed once in medium 199 and fixed with absolute methanol. The coverslips were then stained with Giemsa stain (Harleco) or acridine orange at pH 4.1. The monolayers were then observed under oil immersion using white light or epiillumination with ultraviolet light. The number of gonococci (as diplococci) adherent was estimated by scoring 200-300 cells.

5. Analysis of Attachment Data: Analysis of preliminary experiments using light microscopy suggested that gonococcal colony phenotypes had different abilities to attach to tissue culture cells. These experiments were conducted using large cfu/ml inocula (8.0×10^7 cfu) and Leighton tube coverslip monolayers of cells (2.5×10^5 cells) and visually counting the number of gonococci adherent. Results were expressed as no. of gonococci attached/100 tissue culture cells/ 10^8 cfu inoculum. Ratios (Op colony phenotype attached/Tr colony phenotype attached) were calculated. In general, more of the Op phenotype attached. However problems have been encountered with such assays: 1) visual counts are subject to observer error; 2) only a small subset of the cells in the monolayer are counted; and 3) the distribution of cells on the coverslip and the attachment of bacteria to them is not uniform.

Previous investigations of gonococcal-eukaryotic cell interactions have used single concentration inocula (GC: cell input multiplicity of 50:1, 100:1, etc.). Such experiments, while not inherently incorrect, are not adequate for study of the apparent complexities of attachment. For example, the results obtained with another concentration, given the a priori assumptions that attachment: 1) is a specific process involving a limited number of effectors/receptors; 2) is reversible; 3) is capable of reaching equilibrium; and 4) probably is a multifactoral process involving more than one effector-receptor combination.

The use of ^3H labeled gonococci allows for a more complete estimation of attachment circumventing many of the problems noted with visual count or single high cfu inoculum studies. It also allows analysis of data by the Langmuir isotherm technique as previously applied to study of attachment of streptococci to hydroxyapatite (6,7). We have analyzed our data using the methods of Gibbons and co-workers (6). Our data were standardized for inocula using the ^3H cpm and for tissue culture cell density using the ^{14}C cpm. The results are expressed as the number of gonococci in the inoculum (total) minus the number bound (Q) = number free (C). Ratios of free to bound (C/Q) are developed for each concentration or inoculum of gonococci. The technique allows for estimation of the maximum number of binding sites (N) and the affinity constant of attachment (K_a).

Three programs were developed for a Texas Instruments Model TI 59 program-mable calculator in order to compare data generated in attachment studies. The first program entitled Langmuir analysis processes the raw data (plate counts, ^3H counts, ^{14}C counts, background counts, etc.), normalizes the substrate (tissue culture cells) by conversion of the crude ^{14}C counts of replicates to a common value which allows for the weighting of ^3H counts attached, and enters the data in the regression of C/Q vs. C . This program then generates the slope (m), intercept (b) and correlation coefficient (r); N , the maximum number of interactions; K , the affinity constant; and the Net Effective Binding (NEB), which is defined as N^2K . This analysis is repeated for multiple phenotypes or experimental conditions.

The second program entitled Isotherm Comparisons compares the regression lines generated by the Langmuir Analysis program for the statistical significance of differences between the lines. The analysis is based on the parameters of slope (m), intercept (b) and the variance (S^2) or the points from which the regression was generated. The program generates the F value, compares the F value to the F distribution tables and produces the $P(f)$ and $Q(f)$ for statement of statistical significance. The third program generated the 95% confidence intervals (95% CI) for the regression lines generated by the Langmuir analysis.

6. Buccal Epithelial Cells: Human buccal epithelial cells were collected by scraping of the buccal mucosa with tongue blades and then washing the surface of the blade with Dulbecco's PBS, pH 7.2. For visual examination, the buccal cells were obtained from a donor with very low levels of attached normal flora. For experiments using buccal cells plus ^3H -labeled bacteria, a pool was made from buccal cells obtained from 4-5 individuals. Cells collected in PBS were washed two times with PBS by cycles of vortexing and centrifugation to a cell pellet. Washed buccal cells were then suspended in medium 199 and either the standard inoculum or serial dilutions of ^3H -labeled bacteria were added. Incubation of mixtures was conducted at 37°C for 30 minutes using snap cap plastic disposable tubes and a rotorack. The buccal cell-gonococcal mixtures were centrifuged to pellet the cells. The supernatant was removed and the cells resuspended in medium 199 and gently washed by vortexing. For visual analyses, the buccal cells were spread on glass microscope slides, air dried and gently heat fixed, followed by staining with acridine orange at pH 4.1 or by Geimsa stain. In experiments using ^3H -labeled bacteria, the inoculum, the wash, buccal cells in the cell pellet were counted as ^3H cpm. Plate counts were used to determine the ratio of ^3H cpm to cfu and analyses were done using the cpm/cfu determinations.

7. Uterine Cervix and Fallopian Tube Explant Tissues: Uterine cervix and fallopian tube specimens were obtained from fresh surgical pathology specimens of patients undergoing surgery for reasons unrelated to our studies. The specimens were obtained under the auspices of the University of California San Francisco Committee on Human Research.

The tissue specimens were placed in buffered medium and transported to the Laboratory. Under a dissecting microscope, the fallopian tube and cervical stromas were gently removed. For the fallopian tube tissues, relatively thin pieces of ciliated epithelium were obtained. The cervical tissues were thicker, more dense and more difficult to dissect. Cervical strips 1-2 mm thick and 1-5 mm wide extending from the ectocervix 2-3 cm into the endocervical canal could be obtained. We have obtained an adequate number of these specimens to do 1 experiments, but not enough pieces at one time to develop the techniques definitive attachment analysis. It has proven to be very important to us the tissue culture cell system to develop the methods for definitive analysis N. gonorrhoeae attachment and invasion of explant tissues.

The fallopian tube tissues were maintained at 37°C, 5% CO₂ in Eagles ~~basal~~, BSS with 10% fetal calf serum. Active ciliary action was maintained for 2-3 weeks. The cervical tissues were incubated in the same nutrient salt solutions with added antimicrobial agents. The cervical tissues produced mucus and after a few days of explant culture were surrounded by the mucus. Prior to use in an experiment, the mucus was washed from the cervical tissues although we have chosen to use the tissues as soon as possible after they were obtained.

The same methods for preparing inocula, incubation and counting were done for the tissue explant cultures as for the Flow 2000 tissue culture cell experiments.

8. Scanning Electron Microscopy Studies: The procedures for the scanning electron microscopy (SEM) studies are those used in the UCSF SEM Laboratory as outlined below: Tissue culture cells and explant tissues are fixed in 3% glutaraldehyde in Na cacodylate buffer, pH 7.4, for 1 hour at room temperature followed by 24 hours at 4°C. The specimens are then dehydrated by sequential passage through 20%, 40%, 60% and 70% ethanol. Just prior to the critical point drying the specimens are further dehydrated in 90%, 95% and two washes of absolute ethanol followed by drying in a Bomar critical point dryer. The specimens are then mounted and gold coated using a Hummer sputter coater. The scanning electron microscope we have used is a Cambridge S150.

9. Antigen Analysis. For the preliminary studies of N. gonorrhoeae pili and attachment, we have utilized the ethanolamine solubilization - (NH₄)₂SO₄ precipitation method of Brinton, et al (8) to obtain pili. Two (NH₄)₂SO₄ precipitations have been used yielding pili which we estimate to be > 95% pure; three other very faint protein bands (including major outer membrane protein) were seen when the pili preparations were examined using SDS-PAGE analysis. The molecular weights of pilus subunits from isogenic Op and Tr colony phenotypes varied by 400-500 daltons.

A small amount of the gonococcal outer membrane opacity associated protein was kindly supplied by Dr. Milan Blake, Rockefeller University.

B. RESULTS

1. N. gonorrhoeae Growth and Labeling: Using agar grown bacteria and selective sub-cultures we routinely obtain cultures that are 99.9% pure for one colony phenotype as measured by visual examination of colonies growing on the plates used to determine inoculum cfu/ml. The agar grown cells labeled with ^3H to an average of about 1,000 cfu/cpm. There is day-to-day variation, strain and colony phenotype variation in labeling efficiency. The ratio of cfu/cpm has been determined for all experiments allowing determination of the number of cfu adherent to cells in any given experiment by counting the ^3H cpm for each coverslip or tissue preparation.

2. Adherence to Tissue Culture Cells: Our initial experiments were designed to determine an appropriate cell line for use in our assays as well as compare attachment levels of gonococcal phenotypes. This was done by microscopic examination of Geimsa or acridine orange stained coverslips and counting the number of adherent gonococci per 100 cells/ 10^8 inoculum. Using this technique and pairs of gonococcal phenotypes (P++Op vs. P++Tr, P+Op vs. P+Tr, and P-Op vs. P-Tr) the ratio of opaque phenotype attached to transparent phenotype attached was compared using cell lines HeLa, Flow2000, and HEp 2. For each cell line, opaque phenotypes attached greater than the isogenic transparent phenotypes, although exceptions were occasionally observed; in a few experiments the transparent phenotypes bound slightly better than (less than 2-fold) the opaque phenotypes. Similar data was developed for P+ and P- phenotypes, again with opaque phenotypes attaching better than the isogenic transparent phenotypes.

Since the development of data based on visual assessment of attachment was very time consuming, we compared the use of ^3H -adenine labeled bacteria as measured in liquid scintillation with visual counting of stained coverslips. In general, there was good correlation between ^3H counts and visual counts. The correlation was reduced for HeLa and HEp 2 cell lines. This was due to difficulty in counting acridine orange stained bacteria on these cell lines; the HeLa and HEp 2 cells stain more orange than green. Flow 2000 cells stained more green and it was easier to see orange stained gonococci against this background.

During the visual count studies, it became clear that using different inocula affected the number of gonococci attached to the tissue culture cells. Since the results obtained were dependent on inoculum size and this variable was easily controlled, we compared the number of ^3H -labeled gonococci available to attach with the number attached (Figure 1). The relationship was linear with increasing inocula, but reached a point of saturation where adding more gonococci did not increase net attachment, providing short incubation times were used. This suggested a specificity to the attachment process with a limited number of binding sites.

We proposed that the Langmuir adsorption isotherm analysis technique would be a good method for study of gonococcal attachment using multiple inocula at sub-saturation levels. A number of experiments were done to show that the system we developed was valid. These experiments are outlined in the following paragraphs.

Langmuir isotherm analysis requires equilibrium conditions between the number of bacteria bound and the number in the inoculum. Figure 1 shows that saturation was reached by increasing the number of bacteria in the inoculum. Differences in attachment of opaque and transparent phenotypes also were apparent. Repetition of this experiment showed day-to-day variation in the saturation point of the cells, suggesting that the number of effector/receptors varied from day-to-day and/or the affinity constants of the attachment varied. Figure 2 shows the relationship of

gonococcal attachment with time of incubation for one inoculum of F62 P++Op and P++Tr. The percent inoculum bound reached equilibrium very rapidly. Based on data such as this, we selected 30 minutes as the incubation period. Thirty minutes was the time used for the majority of our experiments. However, we believe the optimum attachment incubation time to be 5 minutes; this time interval presents logistic difficulties when doing experiments. Similar data was developed for detachment of bound gonococci using wash-off times from 1-15 minutes and the number of 2 minute washes. Based on this data, we concluded that a single two minute wash of the coverslip tissue culture cells with attached gonococci was sufficient to remove unattached gonococci.

The relationship between the number of gonococci bound and the number of tissue culture cells attached to the coverslip was studied. There was a direct relationship between the number of cells on the coverslip and the number of gonococci bound as determined by isotope and visual analysis. Because of this relationship and the inherent variability in Leighton tube coverslip cell densities, we standardized our coverslips by a 1-hour pulse of ^{14}C L-amino acid mixture to the cells. The uptake of ^{14}C -amino acids was proportional to the length of time of the isotope pulse and the number of cells being labeled. Thus, by having a ^{14}C -amino acid pulse, the tissue culture cell substrate could be quantitated and thereby, standardized allowing for better comparisons of attachment of gonococci to slightly different cell substrate numbers. The data comparing attachment to tissue culture cells by Langmuir analysis has been normalized (standardized) to a given cell density for each experiment.

The growth phase of the bacteria used in attachment studies can have a significant effect on attachment. We compared the attachment of the gonococci grown for different times on agar plates. This is not strictly comparable to log growth phase, but phenotypes can only be well maintained by agar growth and differences in expression of phenotype occur with increased times of incubation. There was a slight decline in attachment for gonococci grown over 12-24 hours incubation. For purposes of our studies, the N. gonorrhoeae cultures were 16-20 hours old when used in attachment assays.

Initial studies using Langmuir analysis focused on comparison of F62 P++Op vs. P++Tr. Figure 3 shows typical data developed on two separate days. In general, the opaque phenotypes attached better than the transparent phenotypes as evidenced by the isotherm slope being less for the opaque phenotype. Day-to-day differences in level of binding were also demonstrated by the differences in Y-intercept.

Comparison of attachment of multiple colony phenotypes (P++Op and P++Tr, P+Op and P+Tr, P-Op and P-Tr) of strain F62 is shown in Figure 4. This figure is a composite of numerous experiments and depicts the overall analysis of attachment by colony phenotype. Day-to-day variation was present in the ranking of the five phenotypes which repeatedly had high levels of attachment. There are four apparent relationships: 1) highly piliated (P++) gonococci attach slightly better than less piliated (P+) gonococci; 2) for piliated (P++ or P+) gonococci opaque colony phenotypes attached better than transparent phenotypes; 3) for nonpiliated (P-) gonococci, the opaque phenotypes attached as well as some piliated gonococci; and 4) the P-Tr phenotypes were attached significantly less compared to the other given phenotypes. To us, this indicates that some factor other than pili is important in N. gonorrhoeae attachment.

The attachment of multiple colony phenotypes of three clinical strains (obtained from male urethral cultures) were compared by Langmuir isotherm analysis (Figure 5). For strains gl412 and gl417, there was a high level of attachment for all phenotypes. Strain gl413 behaved like F62 with the P-Tr attaching poorly compared to the other phenotypes; P-Op attached as well as some piliated phenotypes. The day-to-day variation was apparent and probably reflected changes in the quantity and/or quality of both effectors and receptors with the level of attachment being the net of these variable interactions. However, the data consistently showed that some nonpiliated colony phenotypes can attach to tissue culture cells as well as piliated phenotypes. This may be the most important observation from experiments of the type depicted of bacteria on stained monolayers that recent clinical isolates of *N. gonorrhoeae* have overall higher attachment levels than do common laboratory strains. Analysis of Langmuir isotherms generated from experiments conducted at 37°C, 21°C, and 4°C suggest that the attachment was temperature dependent with reductions in attachment when the assays were conducted at 4°C.

Experiments were conducted with supernatant media from cells incubated for 30 minutes at 4°C, in an attempt to determine if receptors were shed from the cells by cold shock. Data were developed using these supernatants as the dilution medium for inocula of gonococci and then studying the attachment to cells not previously cold shocked. For both F62 P+Op and P+Tr, there was increased attachment using cold shock cell supernatants.

Experiments have also been conducted using heat killed gonococci (60°C for 1 hour); these bacteria attached to Flow 2000 cells as well as living organisms.

Attachment experiments were conducted at the pH's from 5.5 to 8.0 using multiple inocula in Langmuir analyses and single inocula with quantitation by both ³H-cpm and visual counts of stained coverslips. Results are shown in Figures 6-8 using single inocula. The level of attachment was essentially constant for the P++Tr phenotype; however, the P++Op phenotype shows a pronounced increase in attachment at pH 5.5. Visual assessment of similar experiments have shown that results similar to these developed by use of ³H cpm labeled gonococci. There was increased attachment of P++Op with acid pH. Figure 7 shows representative data from similar pH experiments conducted using the Langmuir adsorption isotherm analysis system with multiple inocula. There also was an increase in attachment of P++Op with decrease in pH. The reverse was apparent with the P++Tr phenotype, where the level of attachment decreased with decreasing pH. Many such experiments were conducted; day-to-day variations in the magnitude of the pH effect were noted. Figure 8 shows the summary of pooled data (³H cpm) in a regression model comparing N, K, and N²K (Net Effective Binding). From this type of analysis, it appeared that for the P++Op phenotype, there was an increase in N with increasing pH and an increase in K with decreasing pH. The net effective binding also was increased with decreasing pH. For the P++Tr phenotype, there was less pH effect; the net effective binding (NEB) and N increased as pH increased; however, K increased with a decrease in pH, although the effect was minimal. The summary of pooled data (visual counts) in the same regression model shows overall agreement with the data developed by ³H cpm assessment of attachment.

The reproducibility of the Langmuir isotherm generated on a particular day was tested by simultaneously conducting a number of replicate experiments. The data for F62 P++Op and the 95% confidence interval (95% CI) for the pooled data are

shown in Figure 9. All replicates fell within the 95% CI. In addition, comparison of all six lines one to another by the Isotherm Comparison Program indicated all lines were statistically the same when comparing variance, intercept and slope. Similar data were generated for F62 P++Tr, P+Op, P+Tr and gl278 P+Op, P+Tr, P-Op and P-Tr; only one of 20 experiments yielded a statistically different regression line. These results indicated to us that the data was highly reproducible on a single day; with minimal organism and tissue culture cell variation, there was apparently minimal variation in results. This gives us confidence that the attachment variation observed on a given day reflects meaningful differences in biological variation and is not a result of experimenter variance.

Experiments were conducted to determine if isolated opacity associated protein could inhibit the attachment of F62 P++Op and P++Tr phenotypes by binding to receptors on the cell surface. Pretreatment of the cells with the "opaque protein" enhanced the attachment of gonococci to the tissue culture cells, and both P++Op and P++Tr were affected. However, the enhancement was statistically significant only for the opaque phenotypes (Figure 10).

Genital Secretions Effects on Attachment: Pools of genital secretions were collected from each of two groups of women attending local VD clinics. The first group had a positive history of gonorrhea and/or a recent positive cervical culture. These women had secretions collected by use of Weck Cell tampons about once a week for 6 weeks post-infection. The second group had a negative history and/or a negative cervical culture. Secretions were also collected once a week for about 6 weeks. The secretions of each group were frozen, thawed, pooled and filtered (0.45 μ) prior to use.

N. gonorrhoeae strain F62 P++Op and P++Tr phenotypes were set up in a Flow 2000 cell attachment assay using Langmuir analysis. Organisms were compared with and without the secretions of each group. Results are shown in Figure 11. For both the infection positive and infection negative secretions pools, there was blocking of the attachment of the P++Op phenotype; however, there was potentiation of the attachment of the P++Tr phenotype. For both effects, the difference was statistically significant ($P < 0.001$).

Seminal Plasma Effects on Attachment: Semen was collected from anonymous donors, liquified, and pooled and filtered (0.45 μ) and stored at -70°C prior to use. A standard attachment assay to Flow 2000 cells using F62 P++Op and P++Tr phenotypes and Langmuir analysis was conducted with and without added seminal plasma in the medium. The results are shown in Figure 12. Seminal plasma pretreatment of N. gonorrhoeae for 15 minutes with addition of the mixture to Flow 2000 cells significantly inhibited the attachment of N. gonorrhoeae strain F62 P++Op and P++Tr colony phenotypes ($P < 0.001$). In a number of studies analysis of stained monolayers have shown similar results.

Buccal Cell Attachment: A few experiments have been conducted using buccal cell preparations in Langmuir isotherm attachment assays. As was observed with Flow 2000 cells, the P++Op phenotype had overall greater attachment than the P++Tr phenotype.

Attachment to Cervical and Fallopian Tube Explant Cultures: Experiments were conducted using cervical explant tissues and ^3H -adenine labeled gonococci to assess the cervical anatomic point of attachment. Tissue pieces were carefully embedded in medium 199 +1.2% Noble agar leaving only mucosal surfaces free of agar. Sections were incubated with about 8.0×10^7 cfu of F62 P++Op and P++Tr for 60 minutes, and washed one time with medium 199. The pieces were then cut into equal sized sections and attached ^3H cpm assessed by liquid scintillation spectroscopy. Results are shown in Figure 13. There was a significant increase in attachment of the P++Tr phenotype in the junctional and post-junctional columnar cell areas. Less of the P++Op phenotype attached for all sections of the cervical tissue.

Similar experiments with fallopian tube explant tissues have yielded variable results depending on the technique used (Figure 14). The initial experiments utilized whole pieces of fallopian tube with all surfaces freely exposed to the inoculum. With this technique, P++Op phenotype attached better than the P++Tr phenotype. We have modified the procedure so that only the ciliated epithelial surface is exposed to the gonococcal inoculum. This was accomplished by agar embedding of the pieces with the epithelial surface protected by a piece of plastic soda straw. This created equivalent epithelial surface areas for each piece of tissue, with the stromal tissue embedded in agar and unavailable as an attachment substrate. Using this technique, the P++Tr phenotype attached to the epithelium to a greater extent than does the P++Op phenotype.

3. Scanning Electron Microscopy of *N. gonorrhoeae* Attachment to Human Fallopian Tube and Cervical Explant Tissues: In the initial SEM studies, we have looked at the in vitro attachment of gonococcal colony phenotypes to tissue culture cells and human fallopian tube and cervical tissues in explant cultures. In the fallopian tubes, there was a striking difference in the attachment for different colony phenotypes. Piliated phenotypes appeared to attach more often and visually were more numerous than nonpiliated phenotypes. Transparent phenotypes (P++Tr) attached more than the opaque phenotypes (P++Op), and the attachment was to microvilli rather than cilia.

In the initial SEM studies, several potentially important observations have been made on the attachment of gonococci to cervical tissues. The squamous-columnar junction can be abrupt or more gradual in transition from squamous to columnar cells. The width of the transition zone may be important because gonococci appear to attach most commonly to cells in this zone. Near the squamous-columnar junction, the microvilli on the squamous cells appear to change in morphology from predominantly ridges with small projections to individual longer microvilli. The *N. gonorrhoeae* appear to attach to the longer microvilli. Piliated gonococci attach more than non-piliated colony phenotypes and P++Tr attach more than P++Op phenotypes. The organisms were seen in greatest density on rounded cells in the cuboidal epithelial cell area near the squamous-columnar junction. In the endocervical canal area, the gonococci were seen attached to microvilli. Many organisms were seen attached to cervical mucus when it was present on the specimen, and nonpiliated phenotypes seemed to have an affinity for the cervical mucus.

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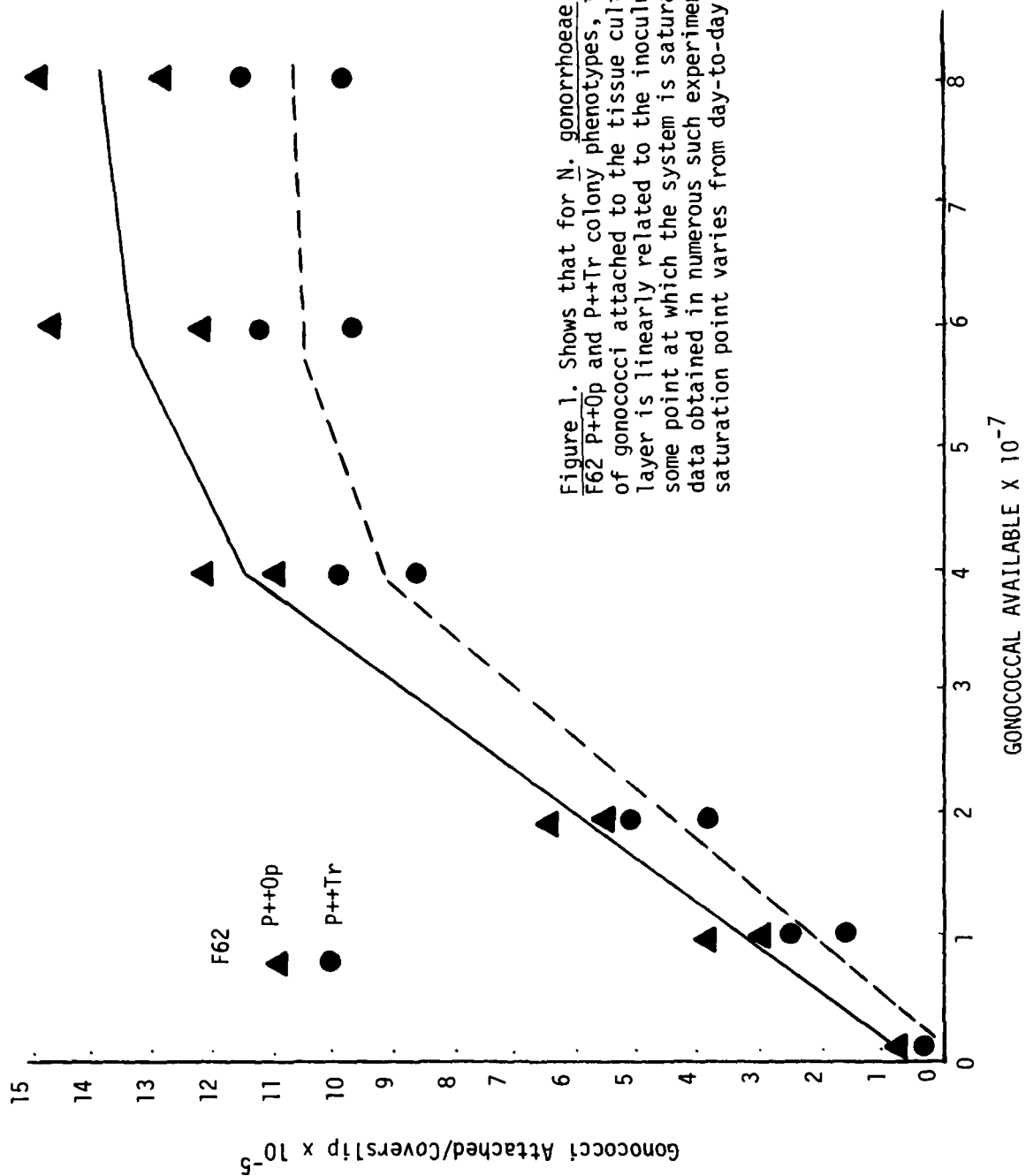


Figure 1. Shows that for *N. gonorrhoeae* strain F62 P++Op and P++Tr colony phenotypes, the number of gonococci attached to the tissue culture monolayer is linearly related to the inoculum size to some point at which the system is saturated. From data obtained in numerous such experiments, the saturation point varies from day-to-day.

Figure 2. Experiment designed to show that *N. gonorrhoeae* strain F62 reaches attachment equilibrium. 50-75% of the attachment obtained in 30 minutes is reached in the first 30 seconds after mixing the gonococcal inoculum and the tissue culture cells. Based on this data, 30 minutes was the standard incubation time.

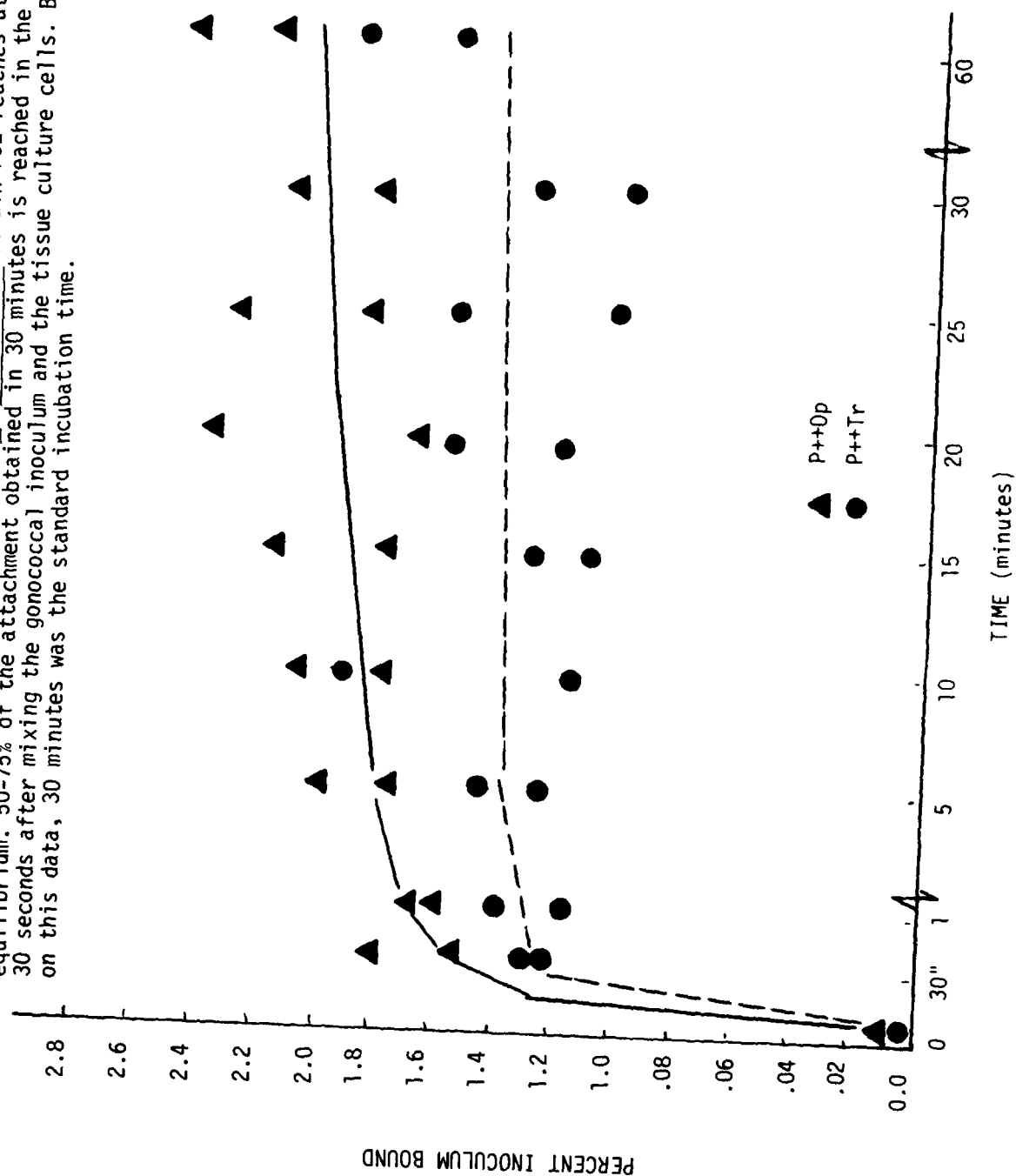
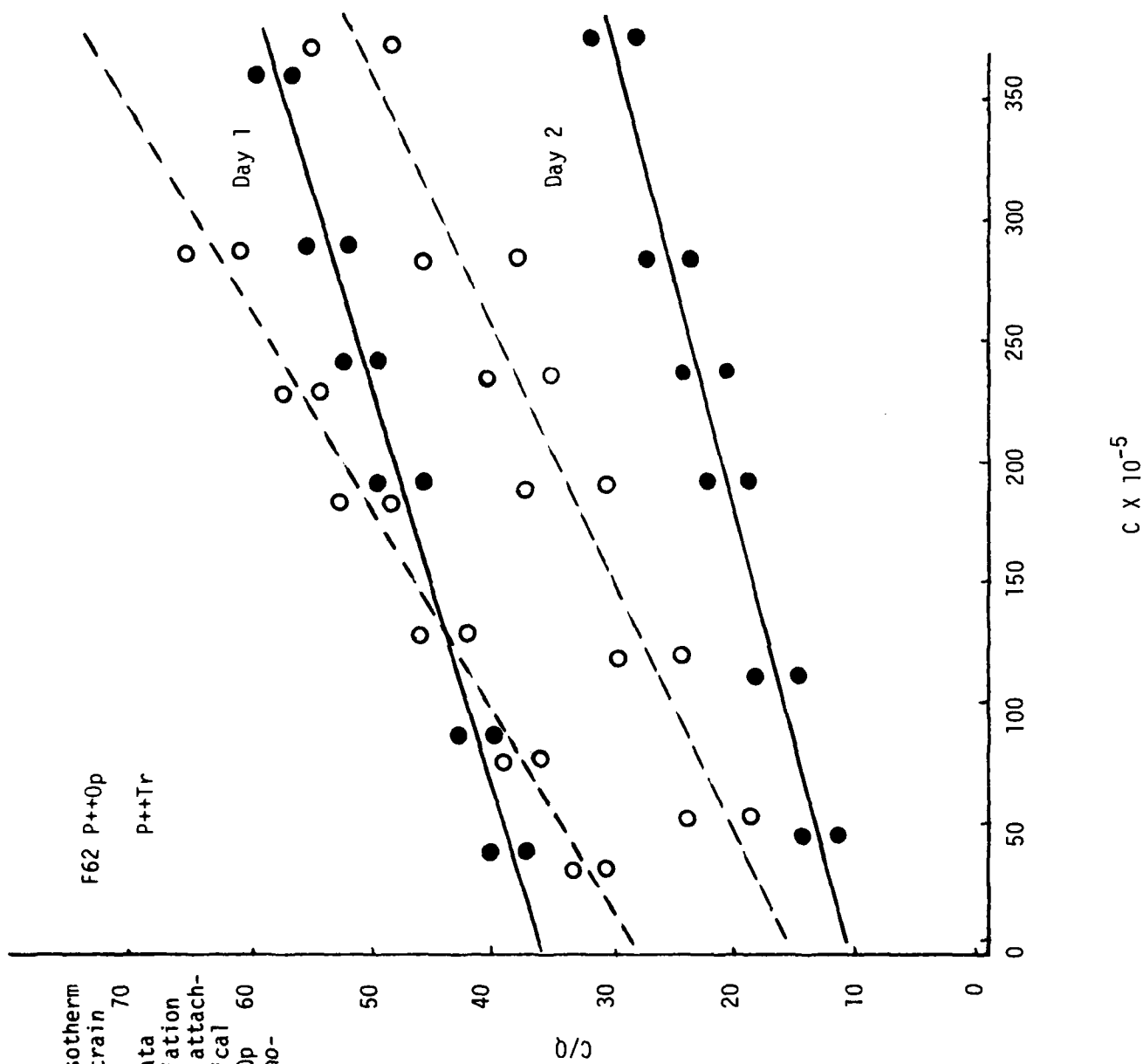


Figure 3. Langmuir Isotherm for *N. gonorrhoeae* strain F62 P++Op and P++Tr colony phenotypes. Data shows day-to-day variation in overall levels of attachment and the two typical relations of the P++Op and P++Tr colony phenotypes.



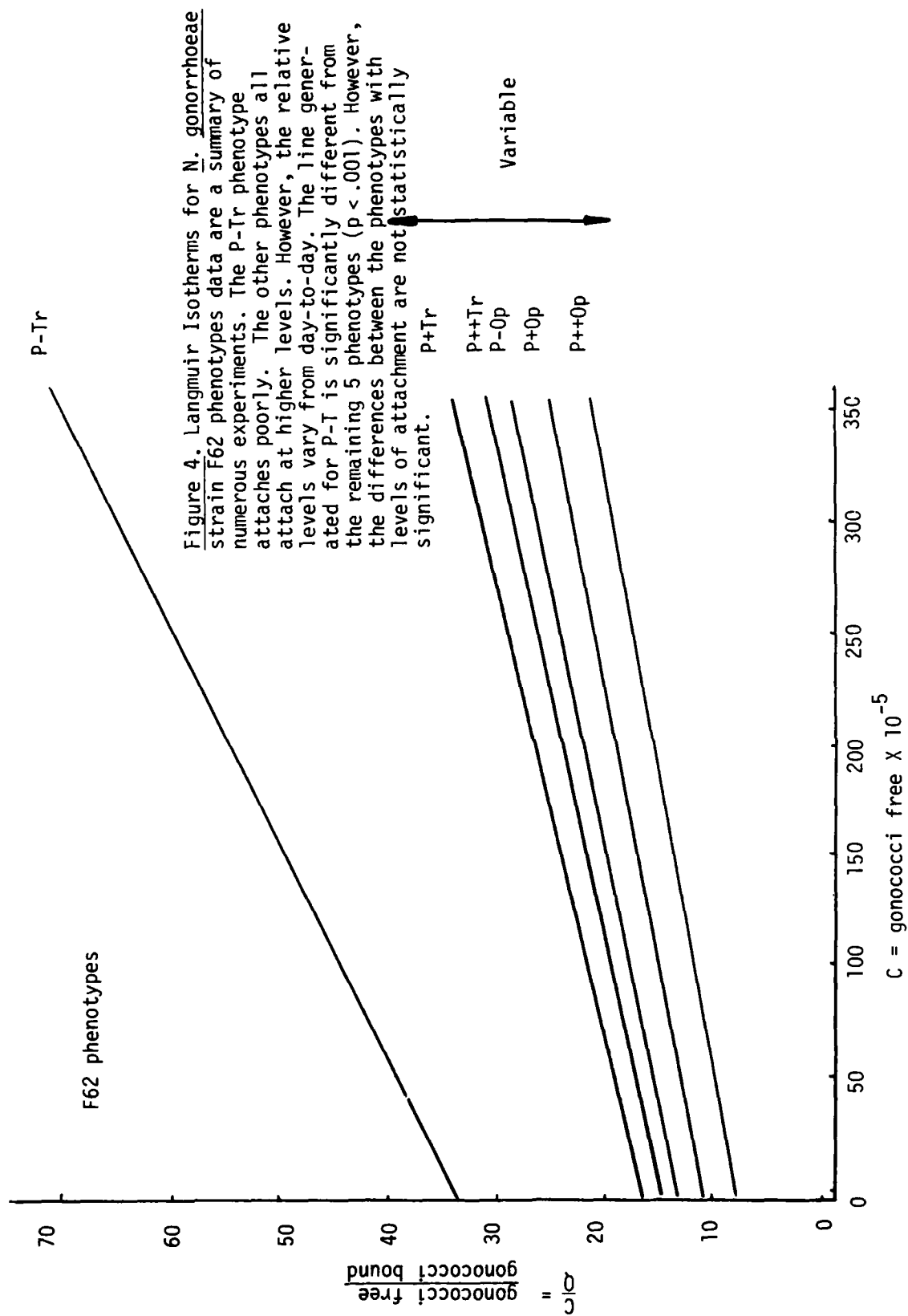


Figure 5. Comparison of Langmuir isotherms generated for three clinical strains of *N. gonorrhoeae* obtained from male urethral exudate cultures. Six phenotypes were rapidly isolated from each strain and the cultures were used in attachment assays with a minimum of subculturing. Data shows strain to strain variation. In general, the clinical strains have overall higher attachment levels than laboratory strains (e.g., g1412). Strain g1413 behaves very similar to the laboratory strain F62. Strain g1417 has a lower overall level of attachment compared to g1413, but does not show the large reduction in attachment of P-T seen in g1413 or F62. The complexity of the relationships is enhanced when g1417 is compared to g1412, where the P-T phenotype attaches quite well.

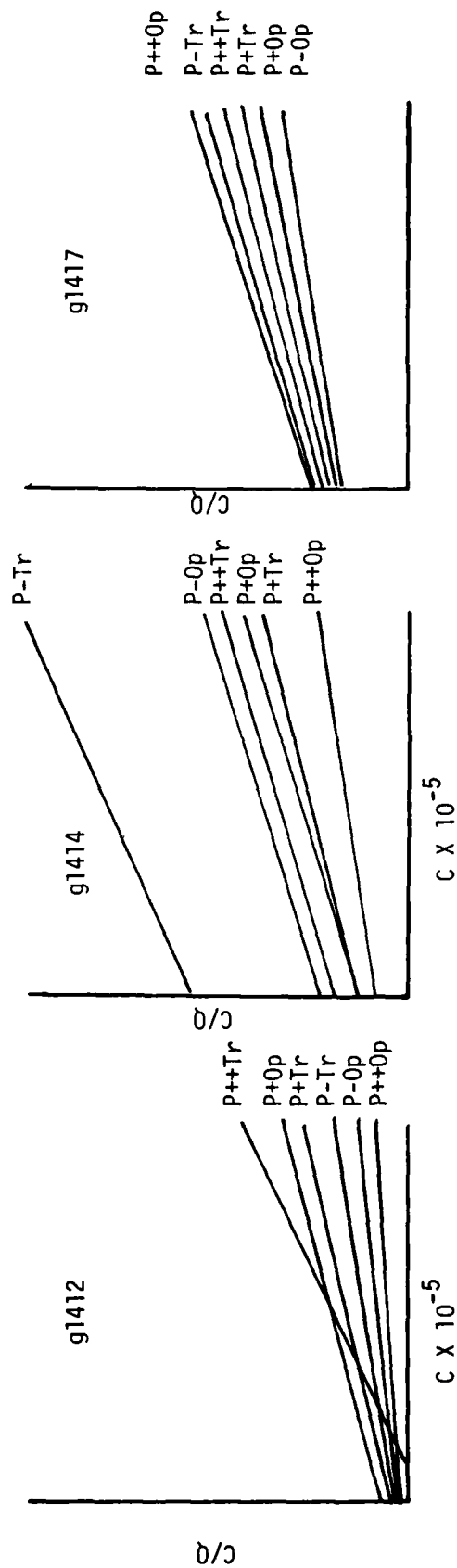


Figure 6. Experiment designed to show the effect of pH on attachment of *N. gonorrhoeae* strain F62 P++Op and P++Tr colony phenotypes. Three experiments were conducted using a single inoculum for each phenotype of about 8.0×10^7 cfu. Data shows that for the P++Op phenotype, the level of attachment increases with decreasing pH. For the P++Tr phenotype, the level of attachment is constant with changing pH.

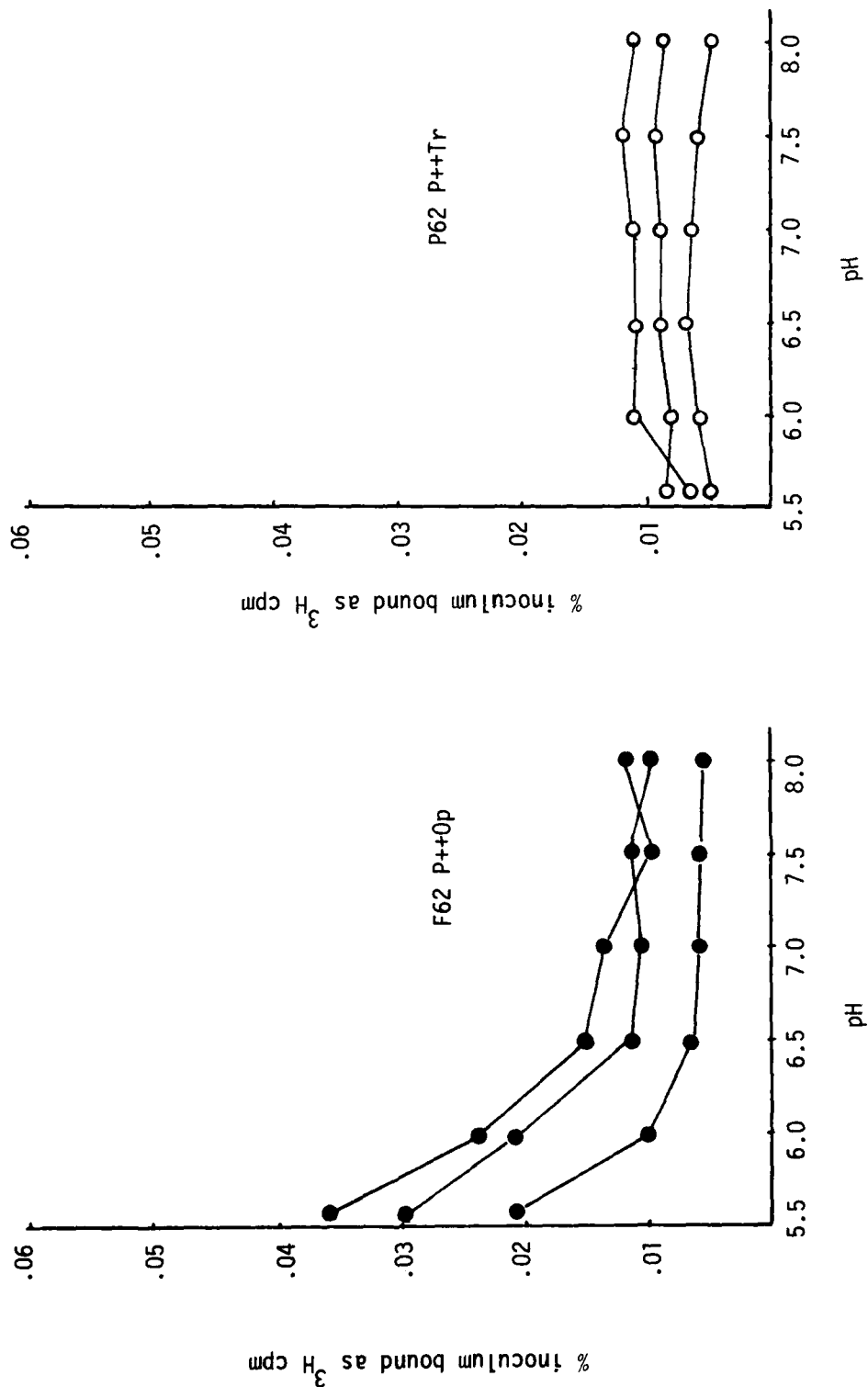


Figure 7. Langmuir Isotherm analysis of attachment of *N. gonorrhoeae* strain F62 P++Op and P++Tr colony phenotypes. Data compares isotherms generated at four different pH's, and shows that overall attachment increased for the P++Op phenotype with decreasing pH. For the P++Tr phenotype, overall attachment increases with increasing pH.

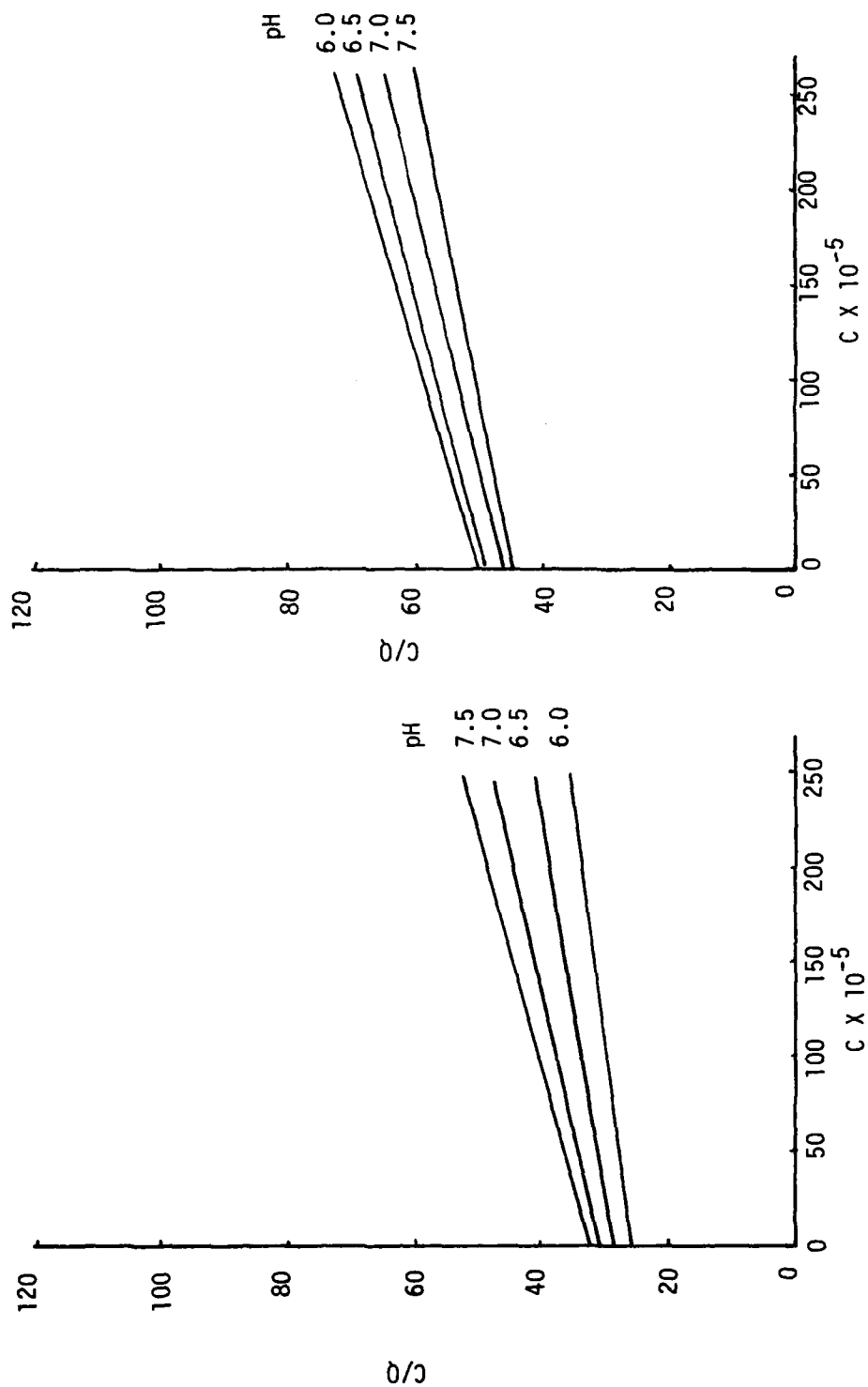


Figure 8A. Regression analysis of data developed in multiple Langmuir Isotherm analyses using *N. gonorrhoeae* strain F62. Data compares the relationship of N, K and NEB to pH; N increases with increasing pH and K increases with decreasing pH. The overall attachment (NEB) increases with increasing pH, suggesting that for the p++ colony phenotype, the number of interactions (N) is more important to the overall attachment (NEB) than is the strength of the attachment (K).

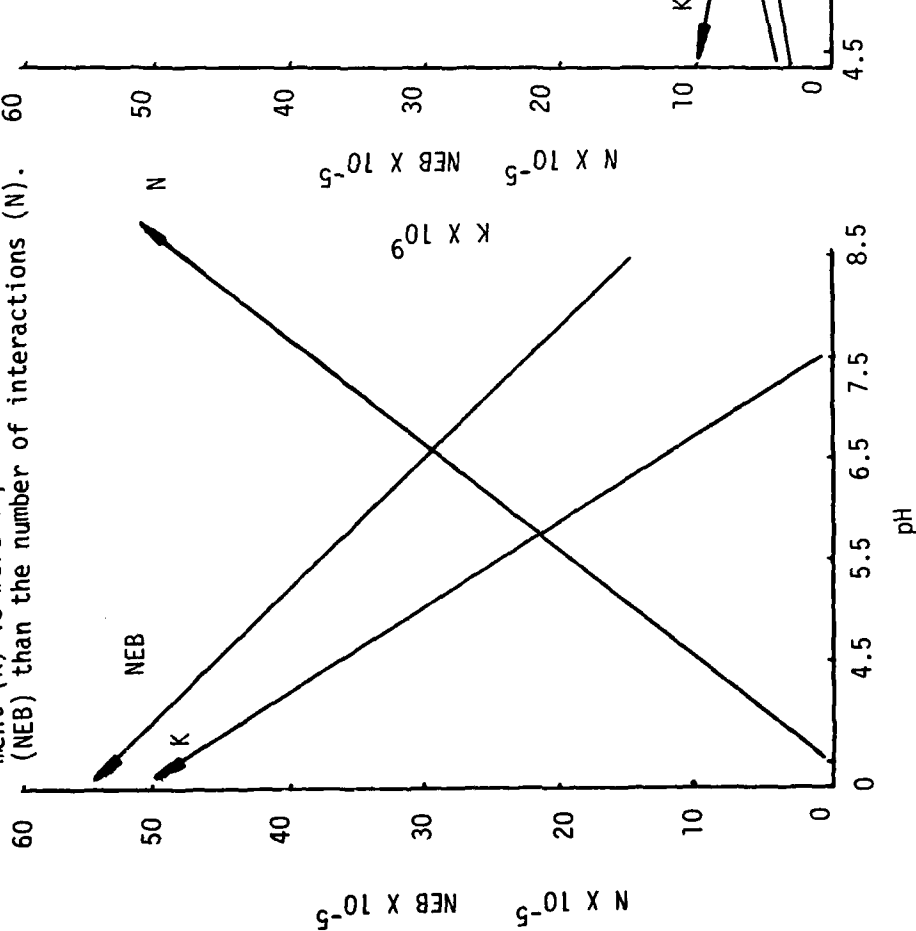


Figure 8B. Regression analysis of data developed in multiple Langmuir Isotherm Analyses using *N. gonorrhoeae* strain F62 p++ colony phenotype. Data compares the relationships of N, K and NEB to pH. N increases with increasing pH and K increases with decreasing pH. The overall attachment (NEB) increases with decreasing pH, suggesting that for the p++ colony phenotype, the strength of the attachment (K) is more important to overall attachment (NEB) than the number of interactions (N).

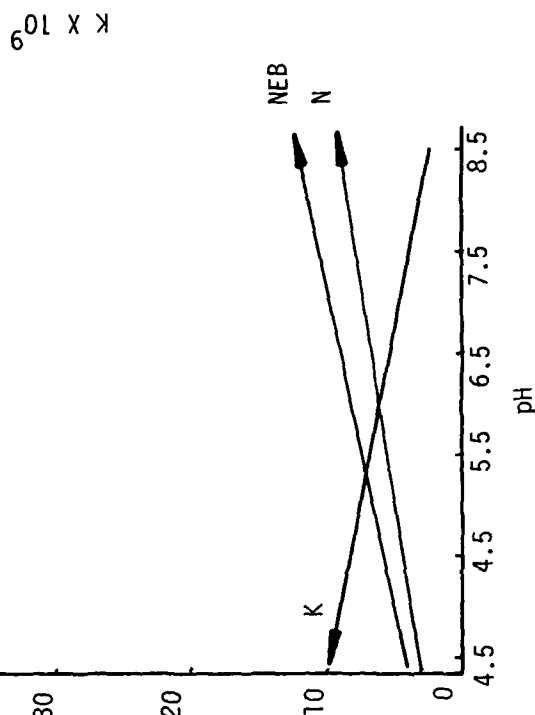


Figure 9. Experiment designed to test the reproducibility of the Langmuir Isotherms. Data compares six replicate experiments with N. gonorrhoeae strain F62 P++0p colony phenotype. All experiments were done on the same day. 95% confidence bands for the regression of the pooled data from all six experiments contain the six individual isotherms indicating a high degree of reproducibility. Correlation coefficient for all six data sets is also high ($r = .97$). The probability that there is no correlation is very low ($p < .001$).

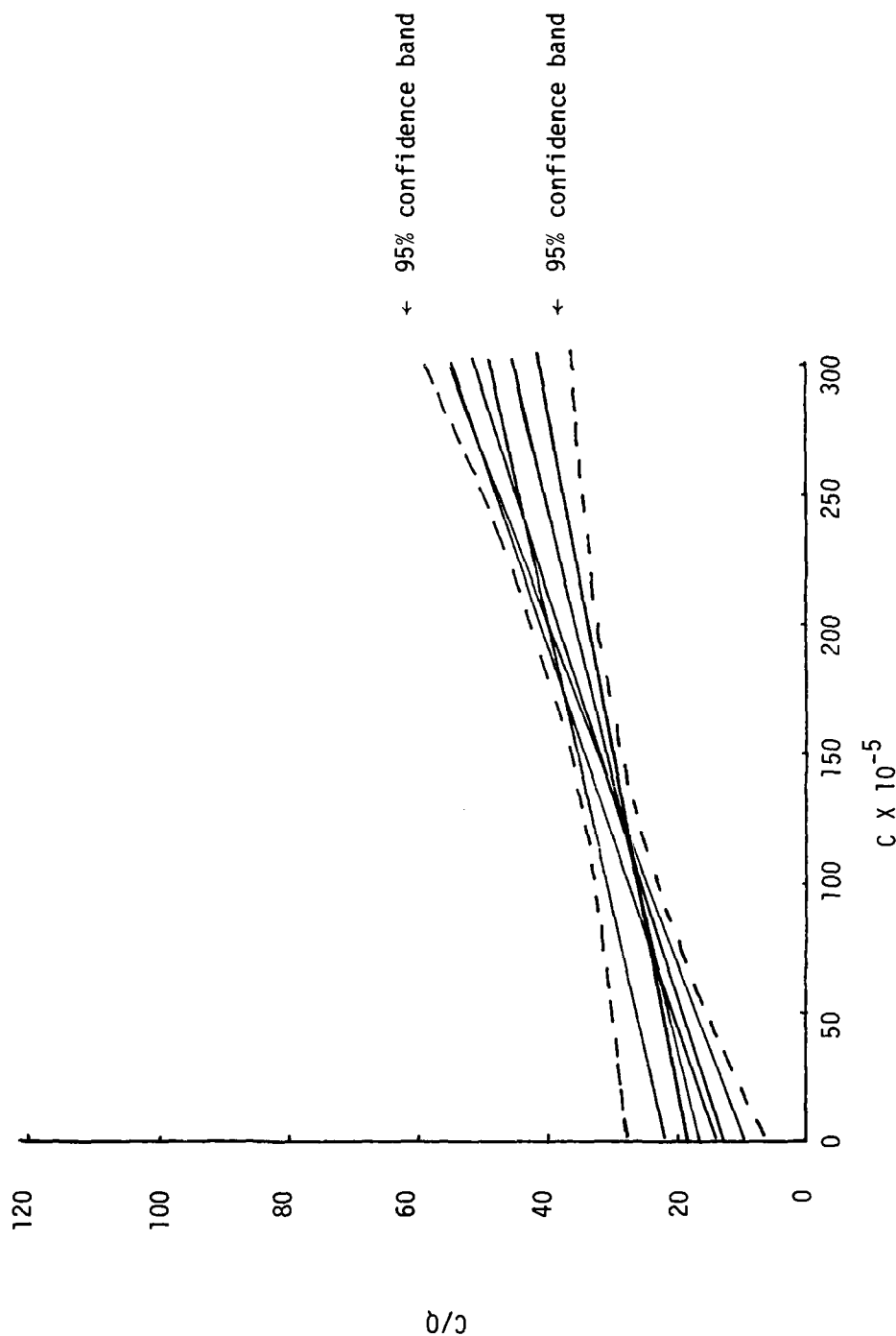


Figure 10. Experiment designed to show the effect of isolated opaque protein on attachment of *N. gonorrhoeae* strain F62 P++Op colony phenotype. Flow 2000 cells were pre-treated with 0.01 or 0.001 $\mu\text{g/ml}$ of opaque protein in M199 at 37°C for 30 minutes, followed by a standard Langmuir Attachment assay. Controls (-opaque protein) were treated with M199 for 30 minutes. Data shows that opaque protein increased the overall attachment of the P++Op colony phenotype. The probability of this difference occurring by chance is low ($p < .90$, $p < .001$). Similar data was developed for the P+Tr phenotype showing a potentiating of attachment by isolated opaque protein.

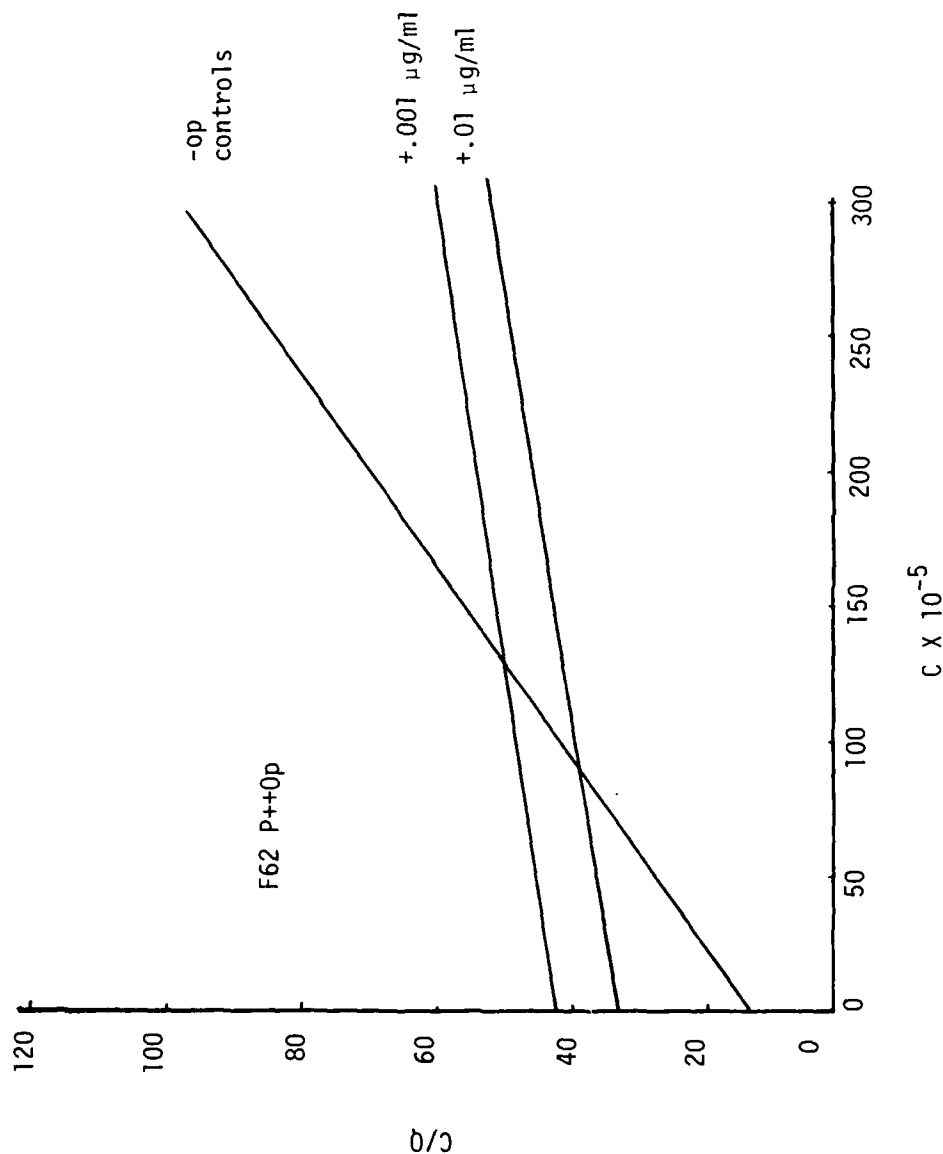


Figure 11. This figure shows an experiment designed to measure the effect of two pools of human cervical secretions on attachment of *N. gonorrhoeae* strain F62 P++Op and P++Tr phenotypes. The first secretion pool (ϕ_1) was obtained from women with a negative history of gonorrhea, and/or a recent negative culture. The second pool (ϕ_2) was obtained from women with positive histories and/or a recent positive culture. These secretion pools were incubated with gonococcal inocula for 15 minutes at 37°C and then added to the tissue culture monolayers for the standard Langmuir attachment assays. The isotherms generated show that for the P++Op phenotype, both secretion pools significantly inhibited the attachment compared to the control (- ϕ). For the P++Tr phenotype, both secretion pools significantly enhanced the attachment. Both the inhibition and enhancement were statistically significant ($P < .001$).

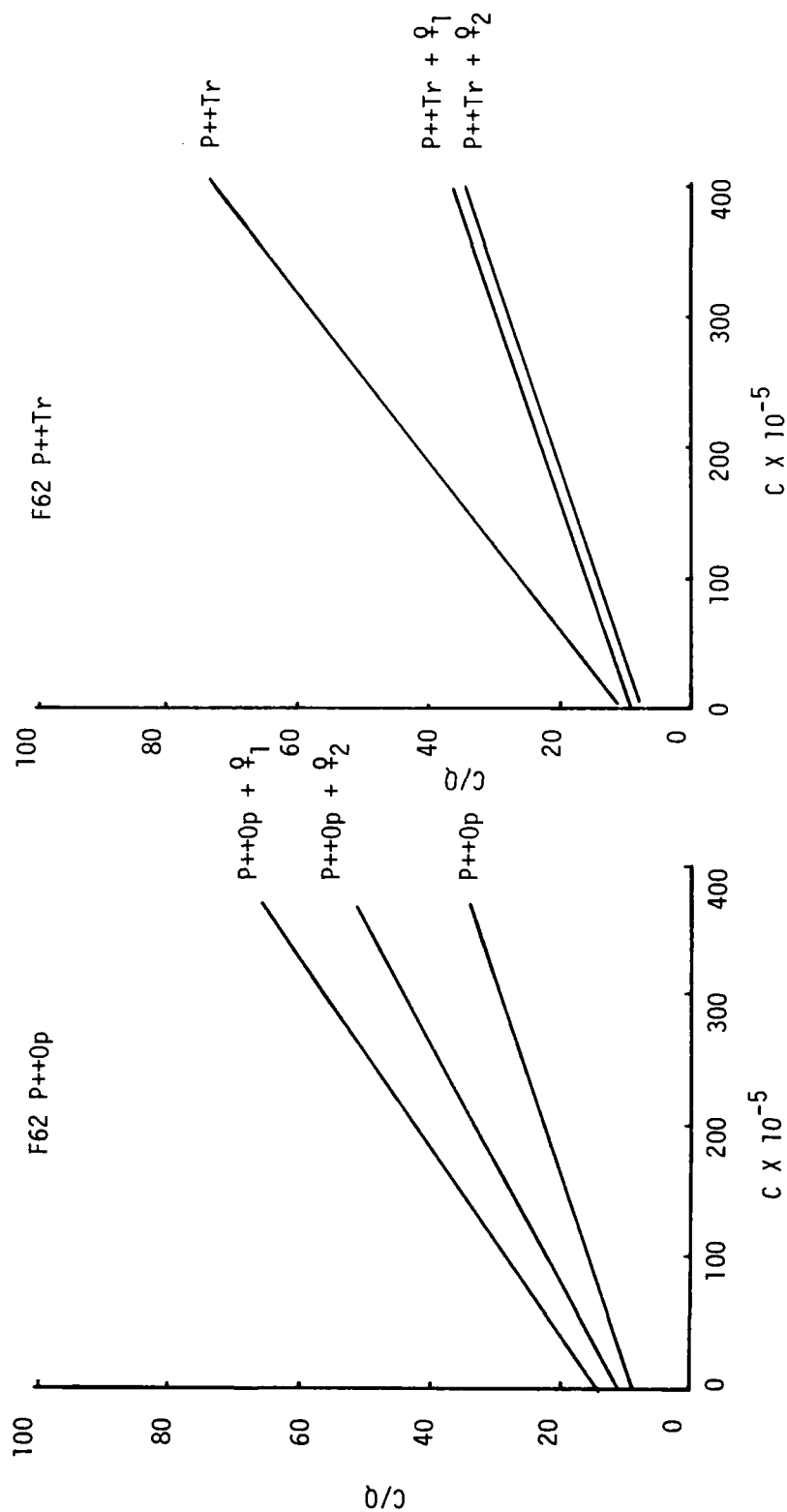


Figure 12. This figure shows an experiment designed to test the effect of a pool of human seminal plasma on the attachment of *N. gonorrhoeae* strain F62 P++Op and P++Tr phenotypes to Flow 2000 cells. The isotherms were generated in the standard Langmuir attachment. The data shows that the seminal plasma treatment (incubated with gonococci for 15 minutes at 37°C then gonococci + seminal plasma added to the cell monolayers) significantly inhibits the ability of both P++Op and P++Tr to attach to the cell substrate. The observed differences between seminal plasma treated gonococci (+ ♂) and control gonococci/cell mixtures (- ♂) were significant ($P < .001$).

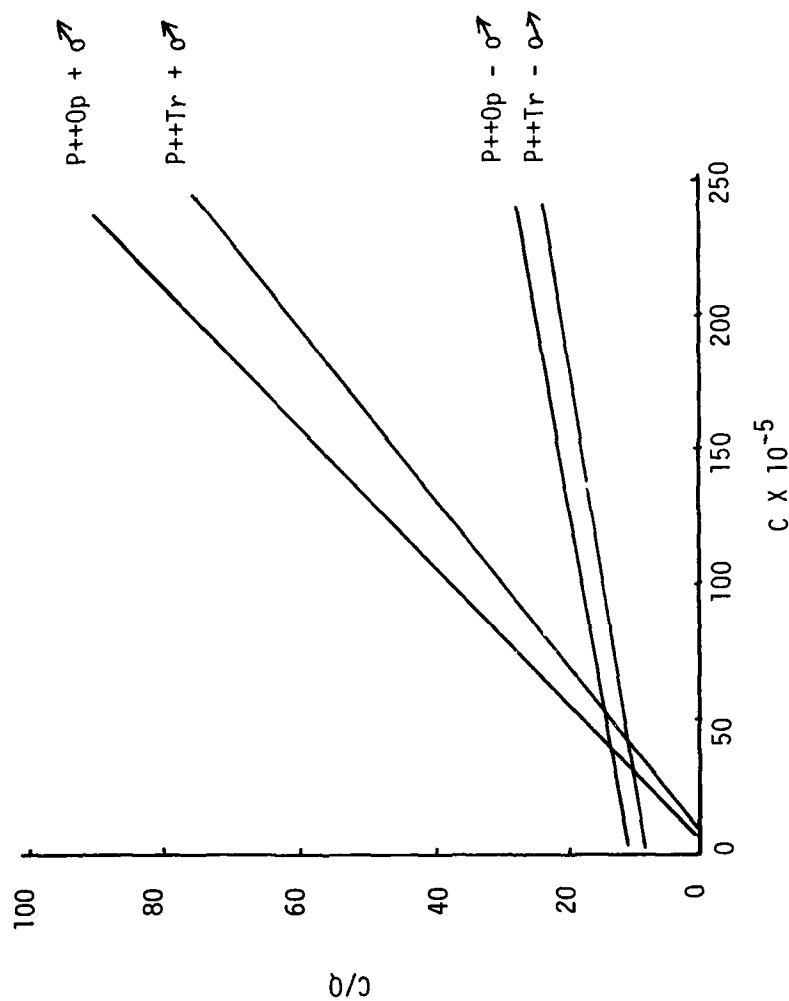


Figure 13. Attachment of *N. gonorrhoeae* strain F62 colony phenotypes P++0, P++I to cervical explant tissues using an inoculum of about 8×10^7 cfu. Data compares anatomical areas of cervical epithelium. The P++Tr phenotype has increased attachment at the squamous-columnar junction.

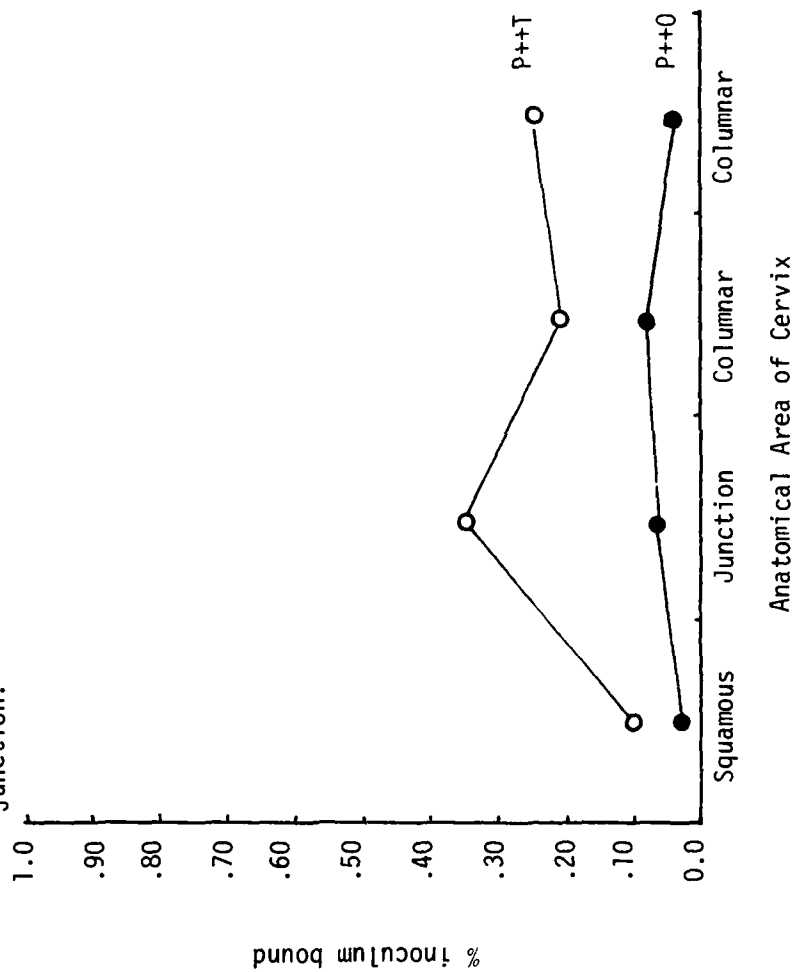
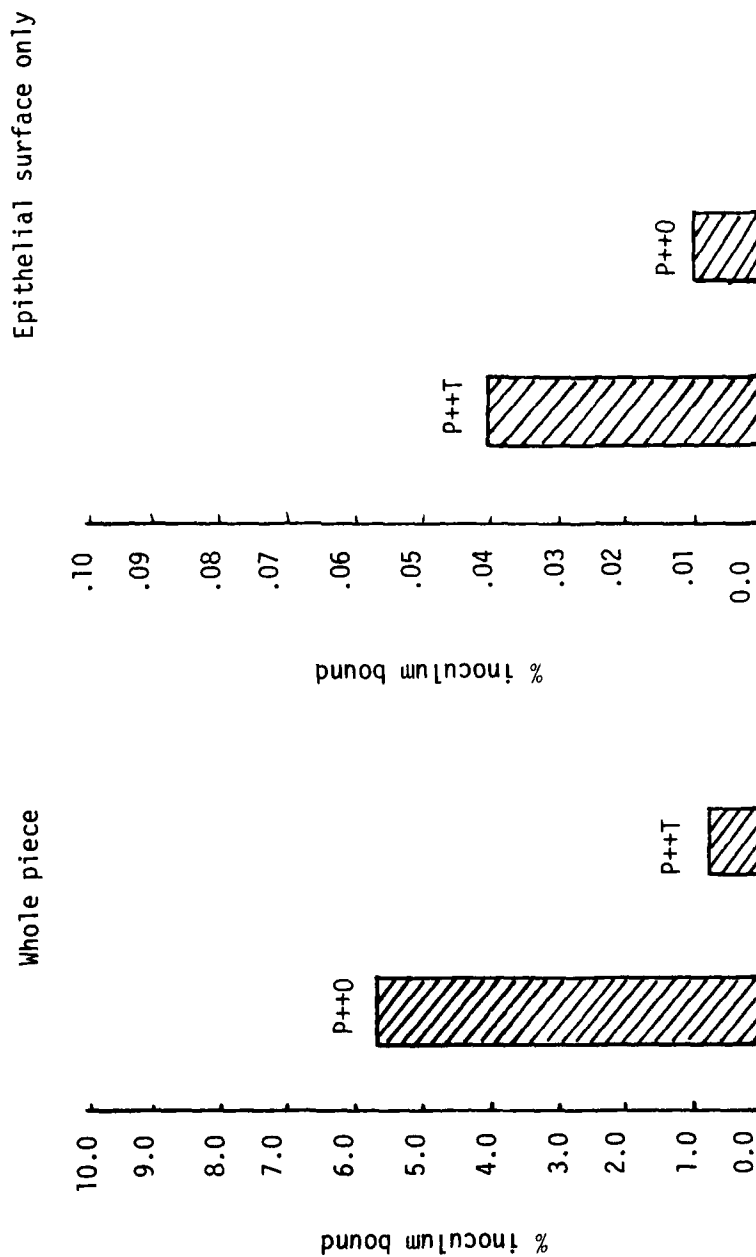


Figure 14. Attachment of *N. gonorrhoeae* strain F62 colony phenotypes P++0, P++T to fallopian tube explant tissue using an inoculum of 8×10^7 cfu. Data shows that P++0p phenotype attaches better than P++Tr phenotype when whole pieces of tissue are assayed. When epithelial surface alone is assayed, the P++Tr phenotype attached better than the P++0p phenotype.



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